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Diversity in function and regulation of MAP kinase pathways

Kendall J. Blumer and Gary L. Johnson

Eukaryotic cells from yeast to humans use sequential protein kinase reactions to regulate complex cellular functions. Equivalent protein kinases in different pathways have significant sequence homologies; however, little crossover in phosphorylation of substrates between pathways normally occurs. Assembly of kinase complexes and discrimination of substrates provide the selectivity of sequential protein kinase pathways to regulate such diverse cellular functions as osmoregulation, cell-wall biosynthesis, growth and differentiation.

CELL-SURFACE RECEPTORS of eukaryotes are used to sense and respond to extracellular stimuli. Commonly, signal transduction pathways in eukaryotes regulate protein kinases that phosphorylate and control the activity of proteins involved in metabolic and transcriptional events. Many signal transduction pathways regulated by different cell-surface receptors are highly conserved in evolutionarily distant organisms. Recent genetic and biochemical studies have revealed conservation in sequential protein kinase reactions involved in two systems – sexual differentiation in yeast and mitogenic signals in man. Several cytoplasmic signal transduction pathways involving sequential protein kinase reactions use a serine/threonine protein kinase referred to as mitogen-activated protein kinase (MAPK; also known as ERK, which stands for extracellular-regulated kinase). MAPK is activated by phosphorylation on both tyrosine and threonine[1]. This reaction is catalysed by a specific threonine/tyrosine-directed kinase, MEK (MAPK/ERK kinase)[2]. In turn, MEK is phosphorylated and activated by the serine/threonine protein kinases Raf and MEK kinase (MEKK)[3,4]. MEK is also activated by Mos[5]. MEKKs are expressed in yeasts and metazoans, whereas Raf and Mos have been detected only in metazoan organisms[6–8].

Receptors with seven transmembrane helices, which are coupled to heterotrimeric G proteins, regulate the MAPK pathway in yeast and higher eukaryotes (Fig. 1). The pheromone receptors in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are coupled to G proteins. The βγ-complex of the pheromone-receptor-coupled G protein mediates activation of the mating MAPK pathway in *S. cerevisiae*[6,7,9]. In *S. pombe*, the α-subunit of the G protein regulates the MAPK pathway[6,8]. Both α- and βγ-regulated effector pathways can stimulate MAPK activation in higher eukaryotes[10]. Metazoans also use tyrosine kinases to regulate the MAPK pathway[11]; yeasts do not have growth-factor-receptor or Src-family tyrosine kinases.

Our current understanding of the MAPK regulatory pathways leaves several questions unanswered. For example, eukaryotic cells express several MAPKs, MEKs and MEKKs which are encoded by different genes. How many MAPK pathways are present in different eukaryotic cells? Do the different MAPK regulatory cascades have distinct or overlapping physiological functions? How is the specificity for regulation of each MAPK pathway achieved? Comparing the characteristics of the MAPK pathways in yeasts and metazoan organisms (Fig. 1) addresses several of these questions.

Independent MAPK pathways regulate different processes

Three signaling pathways employing MAPK homologs have been identified genetically in *S. cerevisiae* (Fig. 2). These pathways control the mating-pheromone response, cell-wall biosynthesis and sensing of hyperosmotic environments[6,7]. Genetic analysis and biochemical studies of the pheromone-response pathway provide a paradigm that indicates that all three systems use sequential protein kinase reactions to phosphorylate and activate the next kinase in the pathway. Among these pathways, homologous protein kinases include: the MEKK-related kinases STE11 and BCK1; MEK homologs STE7, MKK1, MKK2 and PBS2; and MAPK homologs FUS3, KSS1, MPK1 and HOG1 (Refs 6, 7, 12–16).

A striking feature of the three MAPK pathways in *S. cerevisiae* is that they function in mating, cell-wall biosynthesis and osmoregulation independently of one another. Elimination of a specific protein kinase in one pathway by deletion of its structural gene does not affect the function of the other two pathways. For example, cells lacking HOG1 are defective in sensing osmotic stress but are capable of mating[14]. Similarly, cells lacking FUS3 and KSS1 are mating defective but capable of assembling normal cell walls[7]. These findings indicate that significant functional crossover of kinases in sequential phosphorylation reactions in MAPK pathways does not normally occur. The stringency with which the MAPK pathways function in *S. cerevisiae* implies that the constituent protein kinases are highly discriminating in their recognition of substrates. Current genetic data do not rule out crossregulation of the activity of MAPK pathways resulting from different physiological stimuli, but simply suggest that the kinases do not normally cross over in their recognition of substrates.

Discrimination of substrates by the kinases in MAPK pathways is an important regulatory mechanism. Kinases such as cyclic AMP (cAMP)-dependent protein kinase (PKA) and protein kinase

K. J. Blumer is at the Department of Cell Biology and Physiology, Washington University School of Medicine, 660 South Euclid Avenue, Saint Louis, MO 63110, USA; and G. L. Johnson is at the Division of Basic Sciences, National Jewish Center for Immunology and Respiratory Medicine, 1400 Jackson Street, Denver, CO 80206, USA.

C (PKC) recognize linear consensus sequences in polypeptide substrates. By contrast, Raf and MEKK recognize the native MEK protein and even mild denaturation of MEK results in loss of its phosphorylation. The phosphorylation of MAPK by MEK similarly requires a native MAPK protein. Peptides encoding sequences surrounding the tyrosine and threonine in native MAPK are not recognized by activated MEK. The conformational requirements of proteins recognized by Raf, MEKKs and MEKs may restrict the substrates recognized by regulatory kinases in various MAPK pathways. MAPKs also discriminate among substrates. In the mating MAPK pathway of *S. cerevisiae*, FUS3 is required for inducing cell-cycle arrest, but KSS1 is dispensable for this function.

A second mechanism for achieving substrate discrimination appears to involve the formation of macromolecular complexes by components of a specific MAPK pathway. Results of co-immunoprecipitation and two-hybrid experiments that identify specific protein interactions indicate that, in the mating-pheromone pathway of *S. cerevisiae*, STE5 (Ref. 17), a novel protein containing a zinc-finger-like domain, interacts with both STE11 and FUS3 (B. Satterberg and E. Elion, pers. commun.; J. Kranz and E. Elion, pers. commun.; J. Printen and G. F. Sprague Jr, pers. commun.). Complexes involving STE5 and its cognate signaling kinases would allow rapid and selective regulation of the mating-pheromone pathway. Components of other MAPK pathways, such as BCK1, MKK1/MKK2 and MPK1, would not assemble into STE5-containing complexes, excluding their participation in the mating-pheromone response. STE5 is not required for the cell-wall-biosynthesis or osmotic-regulation pathways in yeast. STE5 equivalents have yet to be identified for these pathways or MAPK pathways in other eukaryotic organisms.

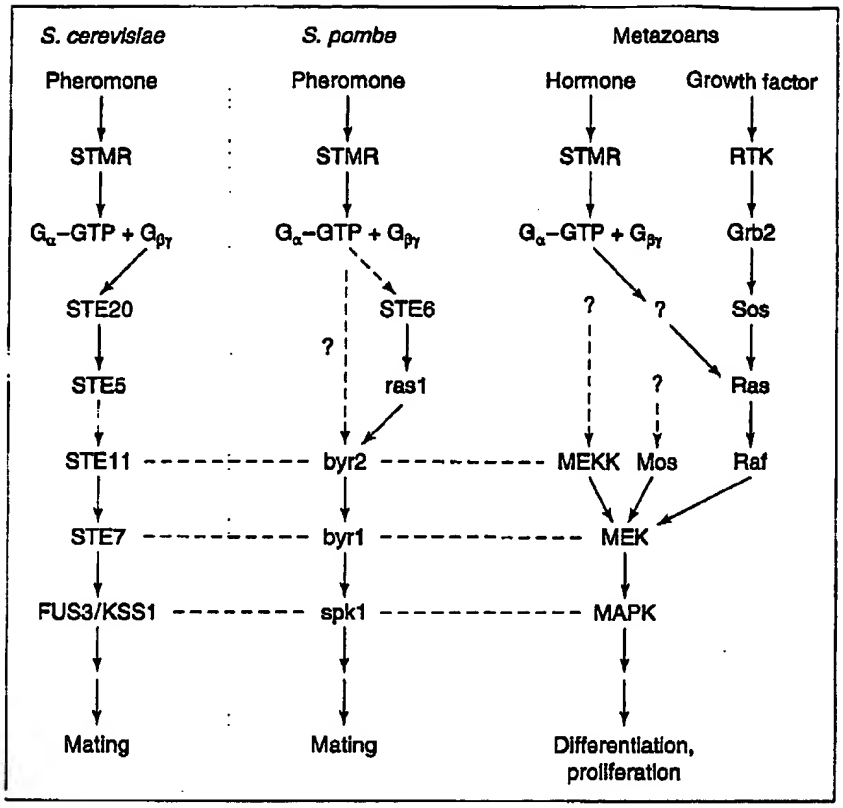

Figure 1

MAPK regulatory pathways in yeast and metazoans. Broken lines identify the homologous serine/threonine protein kinases in the three pathways. STMR, receptors with seven transmembrane helices, which bind pheromone or different hormones in yeast and metazoans. RTK, receptor tyrosine kinases, which bind growth factors in metazoans. See text for details.

Upstream regulators of yeast MAPK pathways

Upstream regulatory protein kinases are involved in activating the mating and cell-wall biosynthesis MAPK pathways in *S. cerevisiae*. The mating pathway is regulated by STE20, a serine/threonine protein kinase that functions genetically between the G protein βγ-complex and STE5. STE20 can phosphorylate STE11 *in vitro*, but whether or not this functionally activates STE11 is unclear (A. Neiman and I. Herskowitz, pers. commun.). It is also unclear whether or not βγ-complexes directly regulate STE20 activity. In the cell-wall biosynthesis pathway, PKC1 (a protein kinase C homolog) functions genetically upstream of BCK1 and phosphorylates the amino-terminal domain of BCK1 *in vitro* (C-Y. Chen and D. Levin, pers. commun.). Activation of BCK1 resulting from its phosphorylation by PKC1 has not been tested. Activators of PKC1, either regulatory proteins or second messengers, are genetically and biochemically undefined at present.

In addition to their roles in controlling MAPK pathways involved in mating and cell-wall biosynthesis, STE20 and PKC1 appear to have additional regulatory functions (A. Neiman and I. Herskowitz, pers. commun.; C-Y. Chen and D. Levin, pers. commun.). Expression of an amino-terminally truncated form of STE20, which is presumably hyperactive, is lethal even when downstream mating-specific genes are disrupted[18]. A lethal phenotype also results in *S. cerevisiae* when *STE20* is disrupted in a *cla4* mutant, which lacks a STE20-related kinase implicated in cell-cycle control (F. Cvrckova and K. Nasmyth, pers. commun.). STE20 and CLA4 therefore have partially overlapping functions. Mutations in *PKC1* cause cell-wall defects that are more severe than those observed with mutations in any of the downstream protein kinases[19], suggesting that PKC1 controls functions in addition to BCK1 activity.

In *S. pombe*, a different upstream regulatory mechanism is used to control a MAPK cascade (Fig. 1). Regulation of the MEKK homolog byr2 appears to involve neither a zinc-finger-containing protein like STE5 nor a regulatory protein kinase like STE20. Rather, byr2 functions genetically downstream of ras1, a low molecular weight GTP-binding protein homologous to the Ras

protein involved in growth regulation and differentiation in metazoans. The byr2 and ras1 proteins interact in the two-hybrid system[20], but whether ras1 directly activates byr2 or regulates activation by other proteins is currently unknown.

The pheromone receptor in *S. pombe* has a predicted seven-transmembrane-helix structure characteristic of receptors coupled to heterotrimeric G proteins. The *S. pombe* G protein α-subunit activates the MAPK pathway in response to pheromone, in contrast with the βγ-complex in the *S. cerevisiae* mating-response pathway. Whether ras1 is activated as a result of G protein α-subunit activation or in parallel with and independently of the G protein is currently unclear.

Regulation of MAPK in higher eukaryotes involves an array of signals that ultimately converge on Raf, which phosphorylates and activates MEK, leading to MAPK stimulation. Receptors coupled to heterotrimeric G proteins, such as those for thrombin, acetylcholine and α-adrenergic agonists, can activate the MAPK pathway[21–23]. These receptors couple to the G proteins G_i and G_q (Fig. 3). Activated α_i, α_q and βγ-complexes can regulate effectors that ultimately stimulate Raf activity. The GTP-bound α_q subunit can regulate phospholipase Cβ (PLCβ) activity, leading to the activation of PKC. Similarly, βγ-complexes selectively activate two isoforms of PLCβ, β_2 and β_3, also leading to PKC activation. PKC directly phosphorylates Raf, but two reports are at odds as to whether or not this results in Raf activation[24,25]. In T and B cells, PKC activation stimulates the loading of Ras with GTP[26,27]. The action of PKC appears to be at the site of the Ras exchange factor, which catalyses the dissociation of GDP, allowing GTP to bind to Ras. In these cells, PLC activation could activate MAPK pathways by this mechanism.

Activation of Ras by heterotrimeric G proteins also occurs by PKC-independent mechanisms. G_i can activate the MAPK pathway by regulating GTP loading of Ras. Regulation of Ras GTP loading by G_i, leading to Raf, MEK and MAPK activation, may be analogous to what is observed in pheromone-receptor activation of the MAPK pathway, and both α_i and βγ may be involved in Ras activation. The steps leading from α_i–GTP and βγ to Ras activation in mammalian cells are currently unknown but appear to involve loading of GTP and not inhibition of the GTPase turn-off mechanism involving GAP.

Tyrosine kinases also activate Raf and the MAPK pathway (Fig. 3). One pathway leading from tyrosine kinases, the epidermal growth factor (EGF)-receptor pathway, involves the SH2 and SH3 (Src homology 2 and 3) domains of an adaptor protein, Grb2, and the Ras guanine-nucleotide exchange factor, Sos. Stimulation of EGF-receptor autophosphorylation results in the binding of the Grb2 SH2 domain to a specific phosphotyrosine-containing sequence in the receptor[28,29]. Via the Grb2 SH3 domain, Sos interacts with the receptor at the cytoplasmic surface of the plasma membrane and catalyses dissociation of GDP from Ras, allowing GTP loading and activation. Ras–GTP interacts with Raf, and this interaction appears to be involved directly or indirectly in Raf activation[30,31].

Because *S. pombe* does not express growth factor receptors or Src tyrosine kinases, a different mechanism apparently activates ras1. Indeed, the *ste6*$^{-}$ gene functions genetically upstream of *ras1*$^{+}$, and encodes a homolog of CDC25, a guanine nucleotide exchange factor for RAS1 in *S. cerevisiae*[32]. The linkage between pheromone receptors, the G protein, ste6 and ras1 is currently unknown.

Raf-independent regulation of MEK

The presence of Raf as a major convergence point for different signals allows the integration of G protein and tyrosine kinase activation of MEK and MAPK. The existence of Raf and its regulation by tyrosine kinase and G protein-coupled signaling pathways does not exclude the existence in higher eukaryotes of MEK kinases similar to the yeast byr2/STE11/BCK1 kinases. The first mammalian homolog of these yeast protein kinases has now been cloned and expressed[3]. Mouse MEK kinase (MEKK) was shown in transfection experiments to phosphorylate and activate MEK-1 independently of Raf. Interestingly, four new mouse cDNAs encoding MEKKs have been cloned, indicating that the number of MEKK isoforms in higher eukaryotes will be similar to or greater than that in yeast. At present, it appears that some, but not all, MEKKs are activated in response to growth factors (G. L. Johnson, unpublished). Some MEKKs will, no doubt, be regulated in response to changes similar to those described in yeast, such as osmotic stress[14]. It is interesting that the mammalian MEKKs have divergent amino-terminal regulatory domains and conserved catalytic domains, suggesting that these isoforms respond to different upstream stimuli. The finding of multiple mouse MEKKs suggests that additional MEKs and MAPKs will be found, as well as new upstream regulators (i.e. kinases like STE20 and proteins like STE5) of these protein kinases.

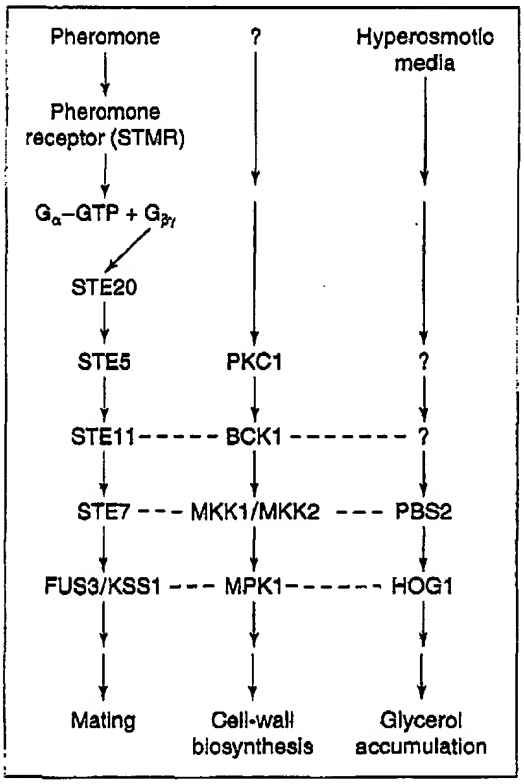

Figure 2
MAPK pathways in *S. cerevisiae*. FUS3/KSS1, MPK1 and HOG1 are MAPK homologs in the mating, cell-wall-biosynthesis and osmoregulation pathways, respectively. Broken lines identify the homologous serine/threonine protein kinases in the three pathways. See text for details.

The downstream effectors phosphorylated by MAPKs in response to MEKK activation are also not defined.

Why have multiple MAPK regulatory pathways? In *S. cerevisiae*, multiple MAPK regulatory pathways respond to different upstream stimuli and recognize selective substrates involved in mating, cell-wall biosynthesis and osmotic regulation. It is predicted that similar regulatory differences for MAPK pathways in higher eukaryotes will also be found. The physiological function and regulation of MEKKs may be quite similar in mammals and yeasts, and do not necessarily require tyrosine kinase signaling for activation.

Turn-off mechanisms for MAPK pathways

Two mechanisms have been defined for negative regulation of MAPK pathways. First, the activation of PKA results in the inhibition of Raf activation in mammalian cells[33–37]. These findings provide one explanation for the long-standing observation that cAMP interferes with the action of growth factors in some cells. The exact mechanism of PKA-mediated inhibition is unclear but occurs physiologically in response to hormonal stimulation of cAMP synthesis. PKA inhibits Raf activation without influencing GTP loading of Ras in response to growth factor stimulation of cells. PKA phosphorylates Raf on Ser43, which inhibits Raf–Ras interaction *in vitro*[36]. PKA did not inhibit phosphoinositide 3-kinase or PLCγ stimulation by growth factors. Thus, the cAMP regulatory system inhibits one step, the activation of Raf, but not other components of the signal transduction network required for mitogenesis in response to tyrosine kinases. The inhibition of Raf activation by PKA is also observed with phorbol-ester- and G protein-coupled activation of the mammalian MAPK system. In many mammalian cell types, such as Swiss 3T3 and PC12 cells, cAMP is not growth inhibitory. PKA regulation of Raf may therefore control pathways in addition to those involved in mitogenesis in different cell types.

Specific tyrosine/threonine MAPK phosphatases also appear to selectively dephosphorylate and inactivate MAPK[38–41]. The physiological importance of these MAPK phosphatases is observed in the mating-pheromone response pathway in *S. cerevisiae*. Cells challenged with a constant level of pheromone initially respond by arresting in the G1 phase of the cell cycle, but they eventually recover and resume mitotic growth. One gene involved in promoting recovery is *MSG5* (Ref. 36). When overexpressed, *MSG5* suppressed G1 arrest caused by a *gpa1* mutation, which deletes the α-subunit of the G protein involved in the pheromone response. Deletion of *MSG5* can blunt recovery from pheromone stimulation. The *MSG5* gene encodes a protein that is approximately 60% homologous to the catalytic domain of CL100, a human tyrosine/threonine protein phosphatase that can dephosphorylate and inactivate MAPK[39–41]. MSG5 can dephosphorylate activated FUS3 *in vitro*, thereby inhibiting its kinase activity. The MSG5 phosphatase activity appears to regulate the mating pathway and additional functions in *S. cerevisiae*. Expression of these phosphatases is induced within an hour or so by pheromone in yeast and growth factors in mammalian cells. These phosphatases are probably responsible for the inactivation of nuclear MAPK. The rapid inactivation of the cytoplasmic MAPK activity that is observed within minutes following stimulation of cells probably involves additional phosphatases.

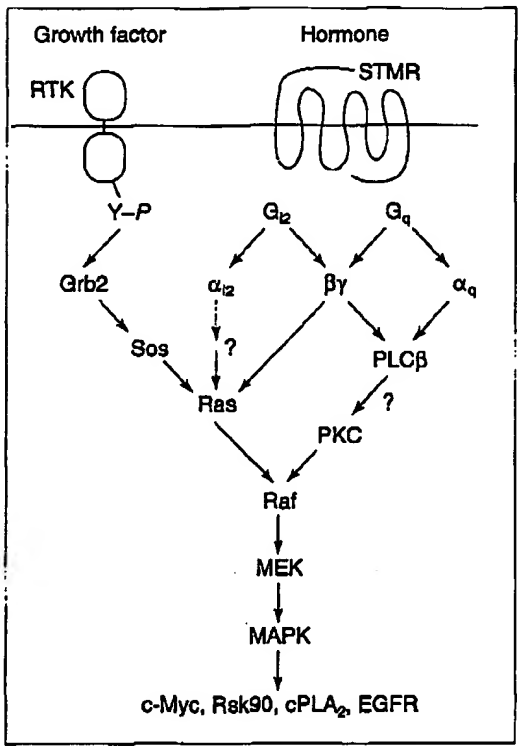

Figure 3
Growth factor and hormone activation of MAPK in metazoans. Autophosphorylation of tyrosine kinase-encoded growth factor receptors recruits Grb2 and Sos to the cytoplasmic surface of the plasma membrane leading to the activation of Ras. STMR (receptors with seven transmembrane helices) coupled to heterotrimeric G proteins can also activate Ras. The intermediates between α_{i2} and Ras are unknown. $cPLA_2$, cytoplasmic phospholipase A_2, EGFR, EGF receptor. See text for details.

Future prospects

Predictably, our current understanding of MAPK regulatory pathways raises many questions. Although yeast MAPK pathways are genetically distinct regulatory units, evidence suggests that activation of one pathway might affect the activity of a second MAPK pathway. Possible mechanisms for crossregulation of MAPK pathways are unknown. It is also unclear whether or not MAPK pathways can be remodeled to function differently in various physiological settings or cell types. Evidence is emerging that a superfamily of MAPK-related proteins and their upstream regulatory kinases exists in metazoan organisms. Do these pathways have diverse physiological roles, as predicted by their apparent number and common expression in different cell types? Answers to these and other questions regarding MAPK pathways and their role in physiological regulation are expected soon.

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Nucleosome positioning and modification: chromatin structures that potentiate transcription

Alan P. Wolffe

The role of the nucleosome in the folding of DNA has often been thought of as purely a packaging one. However, the precise folding of regulatory sequences of genes around the histones within positioned nucleosomes is also important in controlling both the access of transcription factors to chromatin and the transcription process itself. This review highlights these functions by using specific examples of an active and regulatory role for positioned nucleosomes.

POWERFUL GENETIC APPROACHES have defined both the histones and transcription factors as regulators of eukaryotic transcription[1]. Transcription factors are well known for their highly selective recognition of individual DNA sequences; however, histone assemblies can also have selective interactions with DNA[2]. Recent experiments with specific chromatin structures have shown that the selective wrapping of specific DNA sequences around the histones can have a key role in modulating both the activation and the repression of transcription.

At first sight, wrapping regulatory DNA sequences up into nucleosomes imposes many difficulties for their subsequent recognition by transcription factors. Each nucleosome contains approximately 160 base pairs (bp) of DNA wound in two turns around the four core histones (H2A, H2B, H3 and H4) and a variable length of linker DNA between adjacent nucleosomes. The folding of the linker DNA is mediated by a fifth histone, H1 (Ref. 3). Despite this extensive contact with the histones, regulatory DNA sequences can maintain their accessibility to transcription factors by means of nucleosome positioning and modification.

In a positioned nucleosome, histone–DNA contacts start and finish at defined sites (translational position) and DNA is organized such that defined sequences are oriented towards the surrounding solution or the histones (rotational position). Regulatory DNA sequences that are recognized by transcription factors within chromatin are exposed towards solution or are found in linker DNA[2]. Within positioned nucleosomes, regulatory DNA sequences are also in contact with specific histones, so that post-translational modification or dissociation of these individual histone proteins, rather than removal of the entire nucleosome, can modulate the use of these *cis*-acting elements in the transcription process. Moreover, formation of a positioned nucleosome has been found to facilitate transcription on certain promoters by bringing into immediate contact regulatory elements that are separated by DNA segments the length of single or double turns of DNA within the nucleosome. These static loops concentrate transcription factors together for more effective transcriptional activation.

In this review, I will illustrate how the three-dimensional folding of DNA by the histones can be exploited by the transcriptional machinery. I will describe: (1) the activation of transcription from a promoter wrapped into two positioned nucleosomes, where association of a transcription factor with regulatory elements within one nucleosome initiates a chain of events leading to the disruption of this repressive chromatin structure; (2) the formation of a positioned nucleosome between an

A. Wolffe is at the Laboratory of Molecular Embryology, National Institute of Child Health and Human Development, NIH, Bethesda, MD 20892, USA.